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# Telomerase Activity in Hepatocellular Carcinoma and Adjacent Liver Tissues

KEIGO KISHIMOTO, MD, JIRO FUJIMOTO, MD,* MASAHARU TAKEUCHI, MD, HIDENAO YAMAMOTO, MD, TAKAHIRO UEKI, MD, AND EIZO OKAMOTO, MD
*First Department of Surgery, Hyogo College of Medicine, Nishinomiya, Japan*

**Background and Objectives:** Activation of telomerase and stabilization of telomeres are considered necessary for immortalization of tumor cells. Telomerase activity was analyzed in 69 hepatocellular carcinomas and adjacent chronic liver disease tissues. The telomerase activity level was examined in relation to clinicopathologic features.
**Methods:** Telomerase activity was determined by a telomeric repeat amplification protocol. Immature and mature leukocytes were removed from homogenized tissue of adjacent livers using anti-CD45 and anti-CD15 monoclonal antibody-coated magnetic beads.
**Results:** Telomerase activity was detected in hepatocellular carcinomas and leukocytes, but not in liver cells from adjacent chronic liver disease tissues after the separation of leukocytes. All hepatocellular carcinomas displayed telomerase activity, and the activity level correlated with the degree of differentiation ($P = 0.021$) and patient survival ($P = 0.039$).
**Conclusions:** These results indicate that activation of telomerase may be required as a critical step in hepatocarcinogenesis and tumor development, and detection of telomerase activity with removal of contaminating leukocytes may be useful in the characterization or prognostication of hepatocellular carcinoma. *J. Surg. Oncol. 1998;69:119–124.* 

KEY WORDS: telomerase; hepatocellular carcinoma; chronic hepatitis; liver cirrhosis; leukocytes

## INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy throughout the world and is estimated to be responsible for more than 250,000 deaths annually [1,2]. Chronic hepatitis B virus (HBV) infection is a well-documented pathogenic factor in HCC [1,2], and hepatitis C virus infection is also known to be an important risk factor for development of HCC [3,4]. However, the relative contribution of each risk factor is unclear because of their uneven geographic distribution. Tumors arise, at least in part, from genetic alterations that result in the activation of oncogenes and inactivation of tumor suppressor genes [5–7]. Several studies have identified genetic alterations that occur commonly in HCC. These include allelic loss, HBV integration-mediated chromosome alterations, and mutations in the p53 tumor-suppressor gene [8,9]. Although the etiology of HCC is complex, furthering the characterization of the molecular events that underlie the development of HCC is essential.

Each DNA molecule is packaged in a separate chromosome and has one centromere and two telomeres [10]. Telomeres are long stretches of structural proteins and repetitive DNA sequences [$(TTAGGG)_n$], that appear to function in chromosome protection and also anchor chromosomes to the nuclear membrane during replication [10–12]. As the DNA polymerase complex does not replicate the ends of linear DNA, telomeric DNA shortens by 50–200 nucleotides with each cell division [13–15]. This shortening of telomeres has been proposed as the biologic clock that determines the life span of a cell [16]. Cell division usually stops at a certain point, and further divisions result in cell death. Telomere elongation must

*Correspondence to: Jiro Fujimoto, MD, First Department of Surgery, Hyogo College of Medicine, 1-1 Mukogawacho, Nishinomiya, Hyogo, 663-8501, Japan. Fax No.: (81) 798-45-6581.
E-mail: sfujimo@hyo-med.ac.jp
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be necessary for the immortalization of cells [13]. Telomerase, a ribonucleoprotein enzyme, uses its own RNA as a template to add hexanucleotides to the ends of replicating chromosomes [17]. This activity has been detected in human cancers in addition to many immortal cell lines and in germline cells, but has not been demonstrated in somatic cells [18,19]. Strong telomerase activity has been detected in 80–85% of HCC samples, and weak telomerase activity was demonstrable in 9–50% of nontumorous liver tissues [20–22]. Telomerase activity has been detected in leukocytes and hematopoetic cells [23–25]. Homogenized tissues obtained from chronically inflamed liver or HCC contain numerous infiltrative leukocytes, and it is therefore possible that the detected telomerase activity is from the leukocytes as well as the liver tissue.

In this study, we isolated leukocytes from homogenized liver tissue using anti-cluster of differentiation (CD)45 and anti-CD15 monoclonal antibody-coated magnetic beads. Telomerase activity was detected in HCC or separated leukocytes, but not detected in liver cells after the removal of leukocytes. We subsequently investigated the relationship between telomerase activity levels and clinicopathologic features of HCC.

## MATERIALS AND METHODS

### Patients and Tumor Samples

Sixty-nine patients with HCC who underwent surgical resections at our hospital between December 1989 and September 1995 were included in the study. Five patients with metastatic liver cancer from colonic primary tumors were included as normal liver tissue controls. The mean age of the patients was 60 years (range 40–74 years), and 57 of 69 (83%) patients were men. No patient had received palliative treatment such as percutaneous ethanol injection and/or transcatheter arterial embolization before surgical resection. Forty-one of 69 (59.4%) patients had cirrhosis and 28 of 69 (40.6%) had chronic hepatitis, fibrosis, or both. Thirteen of 69 (18.8%) patients were hepatitis B surface antigen positive, and 29 of 69 (42%) patients had anti-hepatitis B surface antibody and/or anti-hepatitis core antibody by enzyme-linked immunosorbent assay (ELISA). Sixty-four of 69 (92.8%) patients were tested with a second-generation hepatitis C virus antibody (Ortho Diagnostics, Raritan, NJ), and 46 of 64 (71.9%) patients showed the presence of hepatitis C virus. A tumor sample and adjacent liver tissues were immediately obtained after the liver resection. The tissues were snap frozen in liquid nitrogen and stored at −80°C. Histologic sections were prepared and stained with hematoxylin and eosin (H&E). The cytological features of the tumors were classified into three groups: well-differentiated, moderately differentiated, and poorly differentiated [26]. The classification of differentiation of these tumors were performed by senior pathologists without knowledge of the telomerase activity in these tumors or other clinical information. Tumor size and number, vascular invasion, metastasis, and patient stage were also evaluated [26]. Stage I refers to solitary tumor ⩽2 cm in diameter without vascular invasion; stage II refers to solitary tumor ⩽2 cm with vascular invasion or multiple tumors limited to one lobe, none >2 cm without vascular invasion, or a solitary tumor >2 cm without vascular invasion; stage III refers to solitary tumor >2 cm with vascular invasion or multiple tumors limited to one lobe, none >2 cm with vascular invasion; stage IVA refers to multiple tumors in more than one lobe; and stage IVB refers to any tumor with distant metastasis. This study includes patienta of all stages of HCC except stage IVB. Twelve patients were classified as stage IVA; none of the patients had apparent distant metastases.

### Cell Lines

The human hepatoma cell line, HuH7 [27], was grown in Dulbecco's modified Eagle medium (DMEM)(Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin sulfate (50 mg/ml), unless otherwise specified. HuH7 was used as a positive control for assessing telomerase activity in clinical samples.

### Isolation of Leukocytes From Adjacent Liver Tissues

Frozen tissue samples (50–100 mg) were dissociated with 5 ml of Hanks' balanced salt solution (HBSS) (Nissui Pharmaceutical, Tokyo, Japan) with 0.05% collagenase (Sigma), 1 U/μl dispase (Godoshusei, Tokyo, Japan), 1 U/μl RNase inhibitor, and 1 mM dithiothreitol at 37°C and then filtered through three layers of gauze. The cell suspension was pelleted at 2,000*g* for 5 min at 4°C. The pellet was washed once in phosphate-buffered saline (PBS) and resuspended in PBS with 0.1% bovine serum albumin (BSA). The anti-CD45 and anti-CD15 monoclonal antibodies conjugated with magnetic beads (Dynabeads®, Dynal A.S., Oslo, Norway) were added to the cell suspension ($3 \times 10^7$ beads/ml) to remove leukocytes. The mixture was incubated for 45 min at 4°C with gentle tilt rotation and placed in the magnetic particle concentrator (Dynal MPC®, Dynal A.S, Oslo, Norway) for 3 min to collect the rosetted leukocytes. The supernatant was carefully removed to another tube and pelleted at 3,000*g* for 6 min at 4°C, while the rosetted leukocytes were attached to the wall of the tube by Dynal MPC.

### Telomerase Activity Assay

We used the telomeric repeats amplification protocol (TRAP) method with modifications [18,28,29]. In brief, frozen tissue samples (50–100 mg) were homogenized in 200 μl of ice-cold lysis buffer [0.5% CHAPS (3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate), 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM

2-aminoethyl ethyleneglycol tetraacetic acid (EGTA), 5 mM β-mercaptoethanol, 0.1 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), 10% glycerol] using Kontes tubes with matching pestles (Molecular Dynamics, Sunnyvale, CA) at 450 rpm. After homogenization, the samples were kept on ice for 30 min. Cell lines and cells isolated by anti-CD45 and anti-CD15 monoclonal antibodies as described above were also resuspended in 25–50 μl of ice-cold lysis buffer and then kept on ice for 30 min. The lysate was centrifuged at 16,000*g* for 20 min at 4°C, and the supernatant was rapidly frozen and stored at −80°C. The protein concentration was determined by the BCA protein assay (Pierce Chemical Company, Rockford, IL); an aliquot of extract (6 μg of protein) was used in the telomerase assay. As a negative control extract aliquots were treated with RNase to a final concentration of 0.2 mg/ml for 20 min at 37°C. Assay tubes were prepared by sequestering 0.1 μg of CX primer [5′-CCCTTACCCTTACCCTTACCCTTA-3′] under a wax barrier (Ampliwax; Perkin-Elmer, Alameda, CA). Each extract was incubated in 50 μl of reaction mixture containing 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 50 μM deoxyribonucleoside triphosphates, 0.1 μg of biotin-labeled TS primer [5′-AATCCGTCGAGCAGAGTT-3′], 0.5 μM T4 gene 32 protein (Boehringer Mannheim, Indianapolis, IN), 5 μg of BSA, 2 U of *Taq* DNA polymerase (Takara, Osaka, Japan), and 5 attograms of internal telomerase assay standard (ITAS), generously provided by Dr. W.E. Wright (University of Texas Southwestern Medical Center, Dallas, TX) for 30 min at room temperature. ITAS is a 150-bp DNA standard that is coamplified with telomerase activity products and is sufficiently long that it does not interfere with the visualization of the telomerase ladder [29]. The mixture was incubated for 3 min at 90°C to inactivate the telomerase activity and then subjected to 31 polymerase chain reaction (PCR) cycles [94°C for 30 s, 50°C for 30 s, and 72°C for 45 s (final cycle, 120 s)]. The PCR products were electrophoresed on a 10% polyacrylamide nondenaturing gel in 0.5 × Tris-borate ethylenediaminetetraacetic acid buffer and transferred to Hybond$^+$ membrane (Amersham International, Amersham, England). The PCR products were detected through the use of a biotin-labeled TS primer with chemiluminescent detection (Toyobo International, Toyobo, Japan) and 30-min exposures to radiography film.

### Semiquantitative Detection

We estimated the telomerase activity by serial dilution as follows: high-detectable at a 1,000-fold dilution (0.006 μg protein/assay); low-detectable at a 100-fold dilution (0.06 μg protein/assay); weak-detectable at a 10-fold dilution or undiluted (0.6 μg and 6 μg protein/assay, respectively); and negative-not detectable.

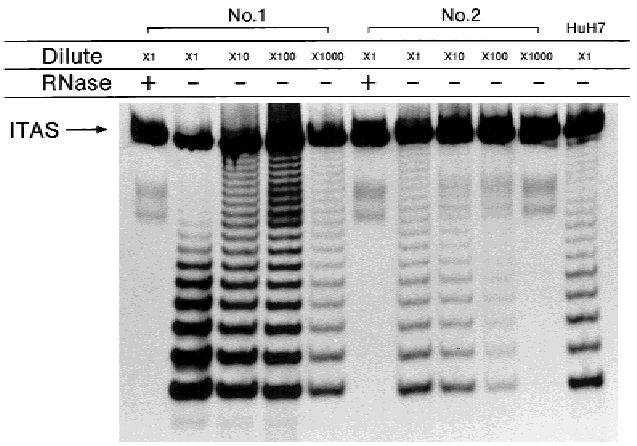


Fig. 1. Representative assay of telomerase activity in hepatocellular carcinoma (HCC). Extracts from HCCs were diluted and used in each assay. Treatment with RNase was also performed before each assay. Representative cases with high (No. 1) and low telomerase activity (No. 2) are shown. HuH7, human hepatoma cell line; ×1, 6 μg protein/assay; ×10, 0.6 μg protein/assay; ×100, 0.06 μg protein/assay; ×1,000, 0.006 μg protein/assay.

### Statistical Analysis

Statistical analysis used the Student's *t*-test and the $\chi^2$-test. The cumulative survival rates were calculated by the Kaplan–Meier method. The log-rank test was used to assess the significance of differences between survival curves. If the *P*-value was $< 0.05$, the difference was considered statistically significant.

## RESULTS

### Telomerase Activity

In HuH7 cells, telomerase activity was clearly detected in a 1,000-fold diluted sample (0.006 μg protein/assay) by telomeric repeats amplification protocol (TRAP) assay, and this provided a positive control for assessing telomerase activity in clinical samples. Sample treated with RNase was used as a negative control in each assay. Telomerase activity was found in all HCCs examined. All HCC demonstrated low or high telomerase activity (Fig. 1). High telomerase activity was detected in 34 of 69 (49.3%) and low activity in 35 of 69 (50.7%) samples. Telomerase activity was not detected in any of five normal livers (Table I).

Telomerase activity was found in 30 of 69 (43.5%) adjacent liver tissues, but they showed very low activity: telomerase activity was detectable only in undiluted samples from adjacent liver tissues. These included 13 cases of chronic hepatitis and 17 of liver cirrhosis. After the separation of leukocytes from adjacent liver tissues, telomerase activity was detectable in the leukocytes, but not in the liver cells from 30 adjacent liver tissues (Fig. 2). It appears that the telomerase activity detected in 30 samples from chronic hepatitis and liver cirrhosis patients was from infiltrating leukocytes.

**TABLE I. Telomerase Activity in Normal and Malignant Hepatic Tissues**

| Samples | Total | Telomerase activity[a] | | | | % positive |
|---|---|---|---|---|---|---|
| | | Weak | Low | High | Negative | |
| HCC[b] | 69 | 0 | 35 | 34 | 0 | 100% (69/69) |
| Adjacent liver tissue | 69 | 30 | 0 | 0 | 39 | 43.5% (30/69) |
| Normal liver | 5 | 0 | 0 | 0 | 5 | 0% (0/5) |

[a]Levels of telomerase activity: weak-detectable at a 10-fold dilution or undiluted; low-detectable at a 100-fold dilution; high-detectable at a 1,000-fold dilution; negative-not detectable.
[b]HCC, hepatocellular carcinoma.

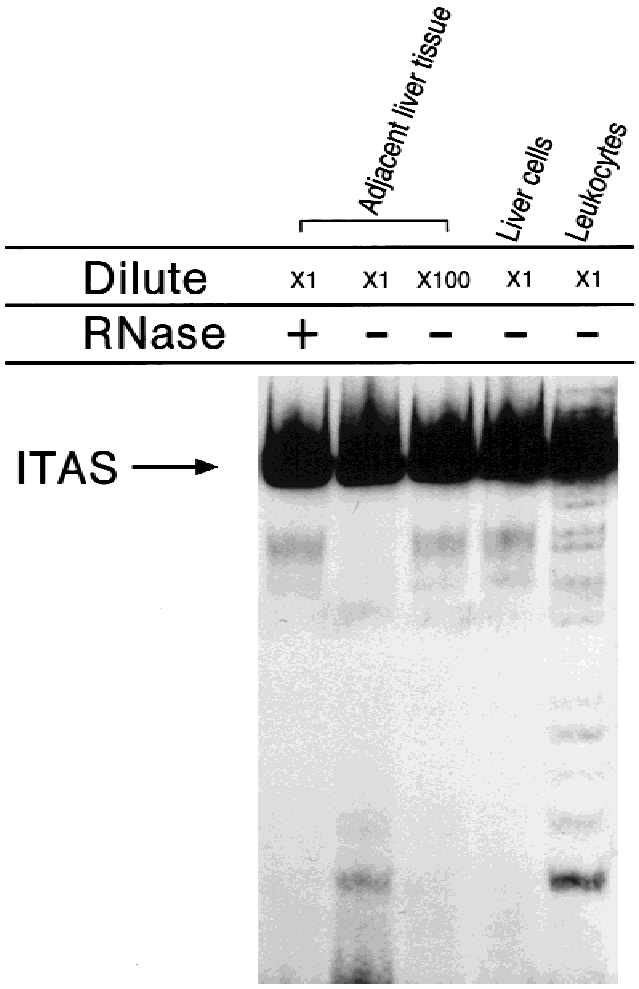


Fig. 2. Telomerase activity in adjacent liver tissue. Telomerase activity was detected in undiluted samples and isolated leukocytes, but not in 100-fold diluted samples and liver cells. ×1, 6 μg protein/assay; ×100, 0.06 μg protein/assay.

## Relationship Between Telomerase Activity in HCC and Clinicopathologic Features

Patients were divided into two groups: low and high telomerase activity. High telomerase activity was associated with poor to moderate differentiation of HCC cells ($P = 0.021$). Telomerase activity was not associated with other histopathologic features (Table II). The 5-year survival rate after hepatectomy was shorter for HCC patients with high telomerase activity as compared with those with low activity (23.5% vs. 62.2%) (Fig. 3). A significant difference between the two groups was seen at 4–5 years after their hepatectomy ($P = 0.039$).

**TABLE II. Relationship Between Telomerase Activity of Hepatocellular Carcinoma and Clinicopathologic Features***

| | Telomerase activity | | |
|---|---|---|---|
| | Low (n = 35) | High (n = 34) | *P* |
| Mean age at operation (years) | 57.8 | 61.4 | 0.129 (NS) |
| Sex (male:female) | 28:7 | 29:5 | 0.562 (NS) |
| HBsAg-positive | 24 | 18 | 0.184 (NS) |
| HCV-positive | 25 | 21 | 0.395 (NS) |
| Degree of differentiation | | | 0.021 |
| Well | 8 | 1 | |
| Moderate | 22 | 22 | |
| Poor | 5 | 11 | |
| Tumor size (diameter) | | | 0.256 (NS) |
| <2 cm | 9 | 5 | |
| >2 cm | 26 | 29 | |
| Tumor number | | | 0.404 (NS) |
| Solitary | 14 | 17 | |
| Multiple | 21 | 17 | |
| Vascular invasion | 22 | 23 | 0.676 (NS) |
| Stage | | | 0.226 (NS) |
| I | 6 | 2 | |
| II | 18 | 14 | |
| III | 7 | 10 | |
| IVA | 4 | 8 | |

*HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; NS, not significant.

## DISCUSSION

In the present study, telomerase activity was detected in 100% of HCC samples, and the activity correlated with the degree of differentiation. These results suggest that telomerase activity may be required as a critical step in the multigenetic process of hepatocarcinogenesis and may also be useful in the characterization or prognostication of HCC.

The CD45 antigen is a common leukocyte marker that is expressed in varying amounts on all cells of hematopoietic origin [30,31], and 99% of lymphocytes, 80–90% monocytes, and 50% of granulocytes can be isolated with

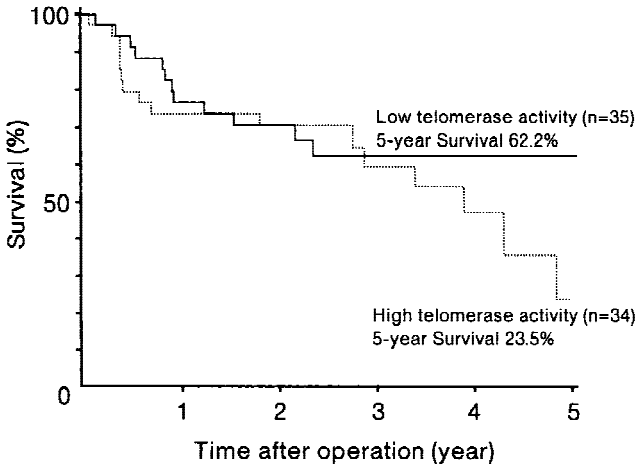


Fig. 3. Survival curves after hepatectomy stratified by the level of telomerase activity in hepatocellular carcinoma (HCC) patients (HCC with high telomerase activity vs. HCC with low telomerase activity). A significant difference between the two groups was seen at 4–5 years after their hepatectomy.

anti-CD45 monoclonal antibody-coated magnetic beads [32]. The CD15 antigen is a surface marker for granulocytes and monocytes [30,33]. All leukocytes can be isolated with anti-CD45 and CD15 monoclonal antibody-coated magnetic beads. We used these antibodies to separate leukocytes from adjacent liver tissues and then assessed telomerase activity in liver cells and leukocytes. In adjacent liver tissues with overall weak telomerase activity, the activity was detected in isolated leukocytes but not in liver cells. We therefore conclude that the weak telomerase activity in adjacent liver tissues was derived from infiltrating leukocytes.

In recent studies, telomerase activity has been measured in various tissues and cell lines and was demonstrable in 85–100% of malignant tissues such as gastric cancer, colorectal cancer, and pancreatic cancer [18,19]. Telomerase activity has been detected in 80–85% of HCC samples [20–22]. Telomerase activity was investigated in total homogenized tumor tissues and/ or liver tissues in these reports. Total homogenized tissue samples contain leukocytes. It has been reported that telomerase activity can be detected in leukocytes [23–25]. According to these reports the telomerase activity may have been detected in leukocytes as well as tumor cells or parenchymal cells. To our knowledge, there has been no report that eliminates the leukocytes from homogenized tissues before analyzing the telomerase activity. The patients with HCC had frequently received palliative treatments such as percutaneous ethanol injection or transcatheter arterial embolization, or both, before surgical resection; consequently, these tissues may have had a necrotic component. The HCC samples in the present study had not been subjected to any palliative treatment before hepatectomy.

Human telomeres, the nucleoprotein complexes at chromosome ends, consist of tandem arrays of TTAGGG repeats bound to specific proteins. In normal human cells telomeres shorten with successive cell divisions, probably due to the terminal sequence loss that accompanies DNA replication [10–15]. In tumors and immortalized cells, this shortening is abrogated through the activation of telomerase, a reverse transcriptase that extends the telomeric TTAGGG repeat arrays [17]. Recently, the rat telomerase protein component 1 gene (TLP1) has been cloned and characterized, but it has also been reported that several tumor cell lines maintain their telomere length without telomerase activity [34,35]. The present study demonstrated telomerase activity in all HCC specimens, and therefore telomerase activity may be necessary for hepatocarcinogenesis. By contrast, telomerase activity was not detected in liver cells from tissue affected by chronic hepatitis, fibrosis, and cirrhosis. Since HCC often arises in cirrhotic livers, activation of telomerase in hepatocytes may be required for hepatocarcinogenesis.

## CONCLUSIONS

Telomerase activity was detected in 100% of HCC tissues and in no adjacent liver tissues affected by chronic hepatitis, fibrosis, or cirrhosis after the removal of leukocytes. The high telomerase activity level correlated with poor to moderate tumor differentiation and poor survival. This study suggests that telomerase activity may be required as a critical step in the multigenetic process of hepatocarcinogenesis and may also be useful in the characterization or prognostication of HCC.

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